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Multilocus sequence typing analysis of *Leishmania* clinical isolates from cutaneous leishmaniasis patients of Iran

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Abstract

Cutaneous leishmaniasis (CL) is mainly caused by *L. major* and *L. tropica* in Old World and might be represented as typical skin lesion(s) or sometimes as a spectrum of atypical manifestations. We applied multilocus sequence typing (MLST) to explore genetic variations of *Leishmania* strains isolated from atypical vs. typical CL patients from Iran. A PCR-sequencing was performed for seven housekeeping genes (*g6pd*, *mpi*, *asat*, *icd*, *6pgd*, *fh*, and *trys*) and genetic diversity indices and phylogenetic relationships were analyzed.

A total of 41 isolates of *L. major* (28/41) and *L. tropica* (13/41) from 21 (51.2%) atypical CL and 20 (48.8%) typical CL cases were included. A set of additional sequences of 41 strains of 17 species of *Leishmania* were retrieved from databases. Different SNP variations were detected and the highest rate of heterozygous sites was found in *g6pd* and *6pgd* genes (6 sites) for *L. tropica* and in *asat* and *6pgd* genes (7 sites) for *L. major* strains. All strains were clustered into 58 unique sequence types (STs) including 17 STs related to 41 strains of *Leishmania* of this study. Concatenated tree clustered all strains in 6 main clades (A to F) including *L. major* (clade D) and *L. tropica* (clade B) strains. Two strains of *L. major* (codes 28 and 42) with highest nucleotide variations were more close to *L. tropica* and were grouped in Clade B. All of the STs were related in clonal complexes by using eBURST with the prediction of founder genotypes.

A high rate of genetic variations and heterozygosity was evident in *L. tropica* and *L. major* strains; nevertheless, there was no significant difference in the diversity of *Leishmania* strains between typical CL and atypical CL groups. This study represents the first successful application of MLST approach to *L. tropica* and *L. major* strains in Iran.

Keywords: *Leishmania*, MLST, Cutaneous leishmaniasis, Genetic diversity, Iran

1. Introduction

Leishmaniasis is a vector-borne disease caused by over 20 pathogen species for humans of the protozoan parasite of the genus *Leishmania*. The clinical manifestations of leishmaniasis are diverse, ranging from a self-healing cutaneous (CL) to a damaging mucocutaneous (MCL) and even to a fatal visceral (VL) leishmaniasis forms (Burza et al., 2018). Leishmaniasis is endemic in 98 countries throughout Africa, Asia, Europe, and North and South Americas. There are an estimated 12 million cases worldwide, with 1.5 to 2 million new cases each year. About 95% of CL cases occur in the Americas, the Mediterranean basin, the Middle East and Central Asia. WHO Eastern Mediterranean Region (EMRO) accounts for 70% of CL cases worldwide (WHO). Self-healing zoonotic CL (ZCL) due to *Leishmania major* and anthroponotic CL (ACL) due to *Leishmania tropica* are two known types of CL that are spread across most parts of Iran. In total, approximately 17 out of the 31 provinces including 88 cities of Iran are endemic foci for ZCL, while ACL is mainly reported from big cities and account for about 20% of CL in the country. The endemic regions in the central and south-western parts of the country (including Yazd, Semnan, Fars, Ilam, Khoozestan, and Isfahan) have the highest rates of CL. According to reports of Ministry of Health, the average incidence rate of CL is 20-40 cases per 100,000 people in Iran and the number of new cases is usually more than 20,000 annually. The epidemiologic pattern of CL in Iran is being affected by appearance of new endemic foci and imported cases from neighboring countries such as Afghanistan and Iraq (Nateghi Rostami et al., 2013). Few cases (100-300 annually) of zoonotic VL (ZVL) caused by *L. infantum* is reported sporadically from all parts of Iran, but in northwestern and southern parts of the country the disease is endemic. The management of leishmaniasis has long been a challenge, since there is no vaccine

available for human leishmaniasis (Keshavarz Valian et al., 2013; Khamesipour et al., 2012) and treatment is not fully effective due to emergence of drug resistance during recent years (Pontes-Sucre et al., 2017).

CL is the most common form of the disease which might be represented as typical skin lesion(s) or sometimes as various atypical manifestations including lupoid leishmaniasis, mimicking lupus vulgaris and common in ACL, erysipeloid, verrucous or xanthomatous nodular presentations in diffuse CL, sporotrichoid lymphatic spread lesions, psoriasiform, and eczematous forms (Firooz et al., 2020). In the Old World, CL is mainly caused by *L. major* and *L. tropica* and rarely by viscerotropic species *L. infantum*.

The different clinical manifestations of CL are dependent upon both intra-species genetic diversity of *Leishmania* and host immune response. However, there is still very limited studies available that have investigated the genetic variation of *Leishmania* spp. in association with clinical features of the disease. Multilocus sequence typing (MLST) method is based on the comparison of partial DNA sequences of a defined set of housekeeping gene targets of the microorganism of choice. The selection of housekeeping genes is based on the assumption that they are under significant purifying selection, which reduces the frequency of deleterious allele in the population and the accumulation of variations by mutation occurs linearly with time, and the resulting genetic distance is proportional to the time of divergence between alleles (Francisco et al., 2009).

For each gene the related sequence is compared to databases, each unique sequence is assigned a number and the combination of alleles at each locus creates an allelic profile and strains sharing the same allelic profile for the set of genes are referred to as sequence types (ST) that identifies a clone. As a highly discriminative typing system, the MLST approach has been widely applied to

different bacterial pathogens since its introduction in 1998 (Perez-Losada et al., 2018; Perez-Losada et al., 2013). However, few studies have applied MLST to *Leishmania* parasite, most of them focused on the taxonomic study of species in Old World (Mauricio et al., 2006; Zemanova et al., 2007)(Zhang et al., 2013) and species belonging to viannia subgenus in New World (Herrera et al., 2017; Marco et al., 2015; Mauricio et al., 2006; Zemanova et al., 2007). Likewise, multilocus sequence analysis (MLSA) has been employed in the taxonomic study of *Leishmania* species in Africa/Eurasia (El Baidouri et al., 2013) and investigation of *L. braziliensis* genetic diversity and outbreaks (Boite et al., 2012; Marlow et al., 2014).

The aim of the present study was to investigate the genetic diversity of seven housekeeping metabolic enzyme genes including glucose 6-phosphate dehydrogenase (*g6pd*), mannose phosphate isomerase (*mpi*), aspartate aminotransferase (*asat*), isocitrate dehydrogenase (*icd*), 6-Phosphogluconate dehydrogenase (*6pgd*), fumarate hydratase (*fh*), and trypanothione synthetase (*trys*) in different clinical forms of CL.

2. Material & Methods

2.1. Patients' characteristics and sampling

This study was approved by the Ethics Committee of Pasteur Institute of Iran. Patients suspected to having CL who were referred to Pasteur Institute of Iran were included in this study for sampling. The patients' demographic data, clinical signs/symptoms, and other information were recorded. All patients were examined by a dermatologist and clinical forms of the CL lesion(s) were determined based on the appearances.

The skin was sterilized, samples were collected by a sterile surgical scalpel from margins of the suspected lesions for parasitological diagnosis and parasite identification. Part of the specimens

smearred onto a glass slide for staining, another part was inoculated into Novy–MacNeal–Nicolle (NNN) biphasic culture media and the last part was collected in PBS vials for DNA extraction and PCR identification.

Exudates on smears were fixed with methanol, and stained with Giemsa, and examined under a light microscope for detection of amastigotes. NNN tubes overlaid with RPMI 1640 medium (Gibco Invitrogen, Carlsbad, CA, USA) were incubated at 25 °C incubator. The liquid phase was examined under the light microscope every other day in order to observe motile promastigotes. For mass production parasites were subcultured on RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 10 mM/L HEPES, 2 mM L-glutamine, 100 U/ml penicillin G and 100 mg/ml streptomycin (Gibco Invitrogen, Carlsbad, CA, USA).

2.2. DNA extraction and Leishmania species identification

DNAs were extracted from lesion exudate specimens by using QIAamp Blood Mini Kit which provides silica-membrane-based nucleic acid purification according to manufacturer's instruction (QIAGEN GmbH, Hilden, Germany). Concentration and purity of DNAs were estimated by measuring the absorbance at 260 nm and 280 nm using NanoDrop One spectrophotometer (Thermo Scientific, Waltham, MA, USA). The DNAs were kept at -20 °C until further use.

Species identification of the isolates was performed by species specific primers with multiplex PCR method targeting kinetoplast DNA and a nested-PCR restriction fragment length polymorphism (RFLP) targeting internal transcribed spacer 2 (ITS2) region as previously described (Nateghi Rostami et al., 2020).

2.3. Multilocus sequencing typing (MLST)

In MLST approach, we performed PCR-sequencing for seven housekeeping genes including glucose 6-phosphate dehydrogenase (*g6pd*), mannose phosphate isomerase (*mpi*), aspartate aminotransferase (*asat*), isocitrate dehydrogenase (*icd*), 6-phosphogluconate dehydrogenase (*6pgd*), fumarate hydratase (*fh*), and trypanothione synthetase (*trys*). The sequences of primers are provided in Table 1. After successful amplification of targeted regions, the pure PCR products were subjected to Sanger sequencing.

The chromatograms of nucleotide sequences of both forward and reverse directions were examined visually and analyzed using the Chromas v.2.6.6 software and edited in BioEdit Sequence Alignment Editor v.7.0.5.3 software (Tom Hall, Carlsbad, USA) . Single nucleotide polymorphism (SNP) variations were identified. Chromatograms were revised in order to detect ambiguous sites (heterozygosity) evidenced by two overlapped peaks in one nucleotide position. Sequences were trimmed and consensus sequences for each gene were obtained through assembly of both strands. Multiple alignment of the nucleotide sequences were done by using the ClustalW program in Molecular Evolutionary Genetics Analysis (MEGA) v.7.0.26 software (Kumar et al., 2016). Sequences were deposited in GenBank under accession numbers MT708905-MT708932, MT700442-MT700457 and MT603308-MT603322.

Sequences were retrieved from NCBI and TriTrypDB databases and comparison with homologous sequences were done using the Basic Local Alignment Search Tool (BLASTn) algorithm. *L. major* MRHO/IR/75/ER strain and *L. tropica* MHOM/SU/74/K27 strain were used as reference strains.

A set of additional sequences of 41 strains of 17 species of *Leishmania* (shown in supporting file 2) were retrieved from NCBI Genome db for the phylogenetic analyses. Analysis of sequences of seven housekeeping genes were done by MLSTest software (Tomasini et al., 2013) and each

unique sequence was assigned an allele number which combined into an allelic profile. Each strain was characterized by a profile of seven allele numbers and assigned a sequence type (ST) number.

In the homozygous strains ST was identical to the haplotype number (H), and for the heterozygous strains was a combination of the two possible alleles.

2.4. Genetic diversity analysis

The indices of genetic diversity of the sequences (Eta, Hd, θ , π and the rate of synonymous to non-synonymous substitutions dN/dS) were calculated for each housekeeping gene in *L. major* and *L. tropica* isolates, using DNA Sequence Polymorphism v.6.11.01 (DnaSP)(Rozas et al., 2017). Nucleotide diversity (π) is defined as the average number of nucleotide differences per site between two sequences. Theta (θ) index is defined as an indicator of mutation rate per nucleotide site per generation. Eta (η) is defined as the total number of mutations and S is the number of polymorphic sites.

2.5. Phylogenetic relationship of the strains

Phylogenetic trees were constructed for each gene marker and the final concatenated tree was constructed for all *Leishmania* isolates as well as all other strains with available genomic data in databases, using Neighbor Joining algorithm with 1000 bootstrap replications by Geneious Prime software.

Phylogenetic relationships among the strains were analyzed through STs which obtained based on the sequences of the seven markers by using eBURST program and PHLOViZ 2.0 software. Clonal complex was obtained based on the prediction of descent from a founder genotype,

establishing the groups of related strains which share at least $(n-1)$ identical alleles at the n loci with other members of the clonal complex.

3. Results

3.1. Demographic and clinical results

In this study, sequence analysis was performed on a total of 41 clinical samples from CL cases who referred to laboratory from central (*L. major*) or South (*L. tropica*) Iran. Among the 41 CL patients, 18 (43.9%) were male and 23 (56.1%) were female. The mean \pm standard deviation (SD) of age of the patients was 41.5 ± 20.02 years, and most of the patients were assigned to the 50–60 years age group.

Based on PCR analysis, 28 of 41 (68.3%) lesional samples were characterized as *L. major* and the remaining 13 isolates (31.7%) were identified as *L. tropica*. The identity of the strains was confirmed with molecular methods (Nateghi Rostami et al., 2020).

Totally, according to skin lesions presentations, 21 out of 41 (51.2%) CL patients were diagnosed as having atypical CL including sporothricoid, eczematoid, lupoid and erysipeloid forms. The rest of the patients (20/41; 48.8%) diagnosed as having typical local lesion comprising papule, nodule, plaque and ulcer. The mean \pm SD size of CL lesions was 20.60 ± 13.31 mm with the minimum of 3.00 mm and maximum of 52.50 mm. While most of the patients had only one skin lesion, about 41% (17/41) developed two or more skin lesions. More than half of the patients (51.2%) were infected in upper limbs (hand, fingers, forearm, arm and neck), 12.2% (5/41) were infected in lower limbs (foot, toe, leg, thigh and back), 14.6% (6/41) were infected on face, and the rest (7/41; 17.07%) had disseminated infection on different parts of the body (Table 2).

As treatment, 36 of 41 CL cases (87.8%) received intra-lesional or systemic Glucantime alone or in combination with cryotherapy. None of the patients had a history of local or abroad travel to known endemic regions during last 6 months before sampling.

3.2. Sequence typing analysis

Seven single copy genes located on different chromosomes (Table 1) were selected as candidates and DNA coding regions were successfully amplified and sequenced.

Analysis of sequences of seven housekeeping genes were done successfully and different SNP variations were recorded based on the chromatograms of the strains in comparison to reference sequences (supporting file 1).

Heterozygous sites were identified based on two overlapped peaks in one nucleotide position in chromatograms. Although most strains were homozygous, almost 17.86% (5/28) of *L. major* and 53.85% (7/13) of *L. tropica* isolates in this study had at least one locus with double peaks. We identified strains with heterozygous sites for all the genes studied (Table 3). MPI was the most homozygous gene in this MLST scheme. Some strains had more than one heterozygous site in a single gene. No tri-allelic site was found.

Examination of sequences variations revealed that in *L. tropica* most heterozygous sites were in *6pgd* and *g6pd* genes with 6 sites of heterozygosity. In *L. major* strains, the highest rate of heterozygous sites was found in *6pgd* and *asat* genes with 7 sites of heterozygosity (Table 3).

Following alignment of sequences and MLSTest analysis, each unique sequence of *Leishmania* spp. was assigned an allele number which combined into an allelic profile and finally all strains were assigned a sequence type (ST) number (Table 4).

A set of additional available sequences of 41 strains of 17 species of *Leishmania* including those of viannia complex from New world were retrieved from NCBI Genome db and used with sequences of the current study in ST determination and phylogenetic analyses (supporting file 2). According to the ST profile shown in Table 4, total 41 strains of *L. major* and *L. tropica* (Table 1) were clustered into 17 unique STs, while total strains of all *Leishmania* species including those retrieved from Genome data bases were clustered into 58 unique STs (supporting file 2). Among all strains most haplotype numbers were observed in *6pgd* gene ($n=37$), while the least haplotype numbers were observed in *asat* gene ($n=23$). About 48.78% of *Leishmania* strains of this study and 24.39% of the total strains were clustered into ST1, the most frequent ST with 100% sequence homology to the related reference strains (Table 4).

3.3. Phylogenetic analysis and clonal complexes

The tree shown in Figure 1a was constructed based on the concatenated sequences of 7 genes using the neighbor-joining method in Genious Prime package.

Totally, *Leishmania* strains isolated in this study together with other strains retrieved from databases clustered in 6 main clades (A to F) comprising CL, MCL and VL species from Old and New Worlds as well as *Leishmania* species of vectors and reservoirs origin. Clade A grouped *L. mexicana* and *L. amazonensis* two main causes of CL in New World. Clade B grouped *L. tropica* and *L. aethiopica* two causes of ACL and disseminated CL (DCL) in Old World. Clade C grouped *L. gerbilli* and *L. turanica* from rodents and sand flies and *L. arabica* isolated in Saudi Arabia. Clade D grouped *L. major* the causative agent of ZCL in the world. Clade E grouped *L. donovani* and *L. infantum* the causative agents of VL in Old and New Worlds. Clade F grouped

L. braziliensis complex including *L. guyanensis*, *L. panamensis* and *L. peruviana* which cause CL and MCL in New World countries. The consensus tree showed strong bootstrap support for the monophyly of the *L. major* group and for *L. tropica* group isolated in this study.

Considering that a large number of unique STs were detected, the BURST algorithm was employed to evaluate the relationships between 58 STs, corresponding to all 82 strains. All of the STs were grouped in clonal complexes, without any singleton (Figure 1b). The analysis figure evidenced the presence ST5 as the main genetic founder of the groups. Other STs such as ST3, ST28 and ST15 are also presented as predicted founder of other ST members in clonal complex. The clonal complex further categorized based on the clinical type of CL, and as shown in Figure 1b, the founder ST5 represented a typical CL strain, while most of the STs corresponding to atypical CL strains were arranged related to each other almost linearly.

3.4. Genetic diversity of the strains

Out of the seven markers evaluated, three genes including *6pgd* and *g6pd* showed greater diversity as represented in different diversity indices in Table 5.

Sequence analysis of the tested genes showed between 9 and 19 polymorphic sites in *Leishmania* isolates. On average, the ratio of nonsynonymous to synonymous substitutions (dN/dS) was 2.720.

Also, the analysis of genetic diversity indices including nucleotide diversity (π) and haplotype diversity (Hd) was performed for the seven markers, with the comparison between strains isolated from typical CL and those from atypical CL groups. The average Hd \pm SD of strains from typical CL group (0.685 \pm 0.239) was not significantly different from that of atypical CL group (0.749 \pm 0.750). Similarly, the average π of strains from typical CL group (0.01201) was

not significantly different from that of atypical CL group (0.01368) (Table 6). Therefore, no significant difference was seen in genetic diversity between typical vs. atypical CL isolates.

4. Discussion

Despite the fact that isoenzyme typing using multilocus enzyme electrophoresis (MLEE) is a well-known widely used approach for identification and taxonomy of *Leishmania* parasites, practical limitations restricts its application to some specialized research laboratories (Schonian et al., 2008; Souheila Guerbouj, 2015). It is a labor-intensive, time-consuming and costly procedure which requires bulk cultures of live parasites. Furthermore, as evidenced in taxonomic study of *L. archibaldi*, a SNP in a gene locus may cause differences in electrophoretic mobility and hence isoenzyme classification of the strains (Jamjoom et al., 2004). Interestingly, growing application of high-throughput molecular techniques has advanced the appropriate identification and taxonomic classification of the parasites (Schonian et al., 2008). MLST is potentially a powerful phylogenetic approach which is believed that would become the new gold standard method for *Leishmania* spp. characterization in the near future (Lauthier et al., 2020). However, few studies have applied MLST to characterize *Leishmania* parasites from Old and New World isolates (Herrera et al., 2017; Mauricio et al., 2006; Tsukayama et al., 2009; Zemanova et al., 2007; Zhang et al., 2013). To our knowledge, previous studies have focused on typing strategies such as restriction fragment length polymorphism (RFLP) and single-stranded conformation polymorphism (SSCP) analyses to evaluate genetic diversity of *Leishmania* strains collected from Iran (Dabirzadeh et al., 2012; Tashakori et al., 2006); so this is the first study that MLST has been applied to clinical isolates of *Leishmania* spp. from the country.

In this study, we have used a profile of seven previously reported housekeeping markers to study genetic diversity of two most prevalent CL causing species, *L. major* and *L. tropica*, and to correlate the genetic variations of the parasite with the clinical outcome of the disease in human. To this end, all samples were collected from the same geographical regions during a three months period. Seven housekeeping genes (*g6pd*, *6pgd*, *mpi*, *icd*, *asat*, *fh*, *trys*) were sequenced from 41 samples of *Leishmania* that were collected from patients with active lesions of either atypical or typical CL. A significant number of haplotypes were detected for each marker and several unique STs were identified among strains.

In MLST scheme, 28 strains of *L. major* and 13 strains of *L. tropica* were collectively clustered into 17 sequence types. Furthermore, genetic diversity indices such as nucleotide and haplotype diversities showed a high genetic diversity of the strains particularly in *6pgd* and *g6pd* genes. *L. tropica* is an anthroponotic parasite compared to *L. major* which is a zoonotic parasite. Since humans are the main reservoir host for *L. tropica* species, these strains are more affected by human behavior; for example it could have been spread through movements of human populations much faster than zoonotic strains, which depend on region-specific animal reservoirs (Dereure et al., 1991; El Baidouri et al., 2013). This may expose *L. tropica* to more rapid genetic variations in its life cycle. Though there is no publication about genetic diversity of *Leishmania* species from Iran using MLST, sequencing of ITS rDNA region suggested high diversity of *L. tropica* with introducing novel haplotypes (Amir HosseinZahirnia, 2018). Similarly, genetic polymorphisms of *L. tropica* from dermal scarping of CL patients in Morocco were analyzed and 13 haplotypes were identified among 125 samples, which suggested the significant genetic heterogeneity of *L. tropica* in the region (El Hamouchi et al., 2019).

The phylogeny based on each marker separately might be associated with some inconsistency among different genes; while, the concatenation based on complete set of genes is a more powerful approach since more genes are included, as previously shown in parasites *Leishmania* (Lauthier et al., 2020) and *Trypanosoma cruzi* (Diosque et al., 2014). In this study, phylogenetic analysis of concatenated sequences from 7 genes showed that all *L. tropica* isolates grouped as a population in clade B along with *L. aethiopica* species, another causative agent of CL. Albeit, two strains of *L. major* (codes 28 and 42) and two strains of *L. tropica* (codes 08 and 47), which were included in the current study, do not form monophyletic groups with others in Clade B, but were intermingled with these strains in the tree. *L. major* isolates 28 and 42 were two strains with highest nucleotide variations and heterozygosity in different loci of all the 7 genes except *mpi*.

The concatenated tree showed strong bootstrap support for the monophyly of the *L. major* group including all strains isolated from CL patients in this study and other reference strains in Clade D. Within the Clade D, *L. tropica* isolate 43 apparently differed from other members of Clade B but closely related and grouped to *L. major* strains.

All strains of *L. donovani* complex including *L. infantum* strains, which cause VL in Old and New World regions, grouped in Clade E. However, within this tree, two strains of *L. donovani* including Ld2001 and Ld39 were separated from others. In some studies it was suggested that *L. donovani* and *L. infantum* strains do not form monophyletic groups but are intermingled in the tree (Jamjoom et al., 2004).

The strains of *L. mexicana* and *L. amazonensis*, which cause CL in New World, comprised Clade A, with strong bootstrap support. All other New World species of *Leishmania*, subgenus *viannia* causing CL and MCL, clustered together in Clade F. Taxonomic studies grouped *L. braziliensis* with *L. peruviana* (Oddone et al., 2009) and *L. guyanensis* (Lauthier et al., 2020) with strong

bootstrap support. There is argument that *L. peruviana* is a subspecies of *L. braziliensis* and it was grouped as a branch inside *L. braziliensis* complex clade (Lauthier et al., 2020).

In this study, neither *L. major* strains nor *L. tropica* strains isolated from CL patients clustered to different clades based on the clinical form of the disease, *i.e.* atypical vs. typical forms. Each of *L. major* and *L. tropica* strains were isolated from CL cases of the same endemic region for ZCL or ACL in Iran, respectively. Geographical origin of the *Leishmania* species seems to determine the position of clusters in the tree rather than other features such as pathogenicity or associated clinical outcome (Zemanova et al., 2007). Phylogenetic analysis of Chinese *Leishmania* species showed that isolates within a clade were subdivided based on the geographical origin, suggesting that intraspecies divergence of strains is correlated with the geographic distribution of *Leishmania* (Zhang et al., 2013). This further demonstrated that the region of a strain isolation is a more important predictor of genetic relationship than the clinical type of disease (Boite et al., 2012; Cupolillo et al., 2003).

Strains of *L. major* isolated from different sources over the past years have shown to be relatively uniform; with three main populations: Central Asia, the Middle East and Africa. In contrast to what is reported for other species which is pointed above, isoenzyme and microsatellite typing approaches showed a weak correlation of *L. major* cluster with the geographical origin, particularly for Middle East strains. It means that genotypes of *L. major* which were found in different geographical areas are similar (Al-Jawabreh et al., 2008; El Baidouri et al., 2013). Based on the result of MLST study, El Baidouri et al. suggested that genotypes of *L. major* from some regions including Iran are in an intermediary position between Africa and Middle East groups (El Baidouri et al., 2013). Genetic heterogeneity of Iranian isolates of *L. major* has been investigated using typing method such as SSCP, sequencing of ITS

regions and random amplified polymorphic DNA (RAPD)-PCR, suggesting the existence of distinct genotypes variants of *L. major* in Iran (Mahmoudzadeh-Niknam et al., 2012; Tashakori et al., 2006).

All of the isolates were grouped in clonal complexes, without any singleton, probably as a consequence of significant rate of mutations rather than recombination. In clonal complex analysis identical STs are related and ancestry is reliably represented by eBURST. Although eBURST has rarely been contributed to phylogenetic analysis in *Leishmania*, it is considered a useful clustering tool to analyze related MLST data, since it reduces the differences exist between *Leishmania* strains (Turner et al., 2007).

Cutaneous leishmaniasis is a polymorphic disease that can represent various clinical manifestations, ranging from typical nodular lesion to different atypical forms (Meireles et al., 2017). In this study, according to genetic diversity analysis, there was no difference between typical CL and atypical CL groups. These findings attenuate any relationship between *L. major* and *L. tropica* genotype and phenotype, indicating that genetic variations of the regions studied here do not seem to be responsible for the variant features and different clinical manifestations of the disease. However, a correlation between clinical outcomes and *Cyt b* sequence variation of *Leishmania* spp from Iranian CL patients was suggested with five distinct haplotypes including three in *L. major* and two in *L. tropica* (Mohammadpour et al., 2019). The presence of CL clinical pleomorphism should be a consequence of epidemiological features, host factors, and the parasite behavior. Several factors other than parasite may cause atypical manifestations of *Leishmania* lesions; including immunosuppression of the host, the number of the inoculated parasites, the site of lesion induction, treatment strategy and bacterial secondary infection of the skin lesions (Firooz et al., 2020; Micallef and Azzopardi, 2014; Nateghi Rostami et al., 2010).

We report here a useful set of seven markers that has been selected with the aim of developing a working MLST scheme for *L. major* and *L. tropica* species taxonomic study. Almost all of these genes which have been studied here, had already been used as a set of MLST markers for other *Leishmania* species (Herrera et al., 2017; Mauricio et al., 2006; Zemanova et al., 2007; Zhang et al., 2013). *Trys* gene is used for the first time as candidate for sequence based molecular typing approach for *Leishmania*. Two of these seven enzyme genes, *6pgd* and *g6pd* revealed highest haplotype numbers in *L. tropica* and *L. major* species. So, they could serve as discriminating markers for phylogenetic analysis of Old World *Leishmania* species. In comparison to New World species, in the study of *L. braziliensis* species the highest value of haplotype diversity was suggested for *alat* and *pgm* markers (Herrera et al., 2017). Haplotype diversity values for *asat*, *g6pd* and *icd* were all higher in the current study compared to those reported for *L. braziliensis* and *L. panamensis* (Herrera et al., 2017).

A significant percentage of *L. major* and more than half of *L. tropica* isolates in this study had at least one locus with double peaks. MPI was the most homozygous gene among the strains, while all the other markers presented more than two sites of double peak per strain. The similar observation has been observed before for the MPI gene (Boite et al., 2012). Heterozygous sites were detected for a few number of isolates particularly in *6pgd*, *g6pd* and *asat* enzyme genes with ≥ 6 heterozygous sites. Heterozygosity can occur due to mutation in one allele, due to genetic exchange between alleles of separate species or coinfection of different species or it can happen intra-species. Mutation is regarded as the possible cause of single heterozygous sites, but heterozygosity at multiple sites within a gene and at several loci can be a consequence of genetic exchange and recombination (Mauricio et al., 2006). Previously, genetic hybrids between clinical isolates of two divergent species, *L. infantum* and *L. major*, has been reported (Ravel et al.,

2006). We could not ignore the possibility of mixed infections which provide the opportunity for genetic exchange (Mauricio et al., 2006). In the case of *L. infantum*, most of the newly discovered zymodemes were reported to be heterozygous, particularly in HIV positive cases who are intravenous drug users and have higher chance of mixed infections (Cruz et al., 2002).

5. Conclusions

While this study has provided evidence of high genetic diversity among *L. tropica* and *L. major* in Iran, more clinical samples are needed to be investigated to explore contribution of genetic variations of the parasite in different clinical features of leishmaniasis in human. The presented MLST approach on phylogenetic study of *L. major* and *L. tropica* strains can be expanded to several other *Leishmania* species in Old and New Worlds.

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Authors' contribution

MH performed the laboratory experiments and sequence analyses and wrote the manuscript draft; MNR conceived the project, performed the genetic analyses, collected the samples and edited the manuscript; RHD contributed in data collection and phylogenetic analysis. AK performed clinical examination and diagnosis. All authors read and approved the final manuscript.

Data statement

The sequences reported in this paper were deposited in the GenBank database under accession numbers MT708905-MT708932, MT700442-MT700457 and MT603308-MT603322.

Declaration of competing interest

Authors declare that they have no conflict of interest.

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Table 1. The sequence of the primers used for the analysis of seven candidate genes in MLST assay

Target gene	Forward (5'>3')	Reverse (5'>3')	Annealing temp	Product size (bp)	chromosome	Ref.
<i>g6pd</i>	ATGTCGGAAGAGCAGTCT	TCACAGCTTATTCGAGGGAA	52°C	1690	34	(Zemanova et al., 2007)
<i>Mpi</i>	ATGTCTGAGCTCGTAAAGCT	CTACCTGTCGCTCAAGTC	54°C	1267	32	(Zemanova et al., 2007)
<i>Asat</i>	ACGAGCGCCGTCCGCAA	TTCCCACATCCACCAAGC	59°C	890	24	(Mauricio et al., 2006)
<i>Fh</i>	AGCGTCTTGTGTTCCCA	GAGCCCGTGTAAGGAGGC	54.5°C	1780	29	(Zemanova et al., 2007)
<i>Icd</i>	ATGTTCCGCCATGTTTCGGC	TTACGCGCTCATCGCCTT	56°C	950	10	(Zemanova et al., 2007)
<i>6pgd</i>	ACCAATCTCCAGAGCCTCCT	TTCGGCTTCGACAACGATCA	58°C	706	34	(Ceccarelli et al., 2018)
<i>Trys</i>	TACGTAGTCGTCACCCACCT	TGGTGCGTAAGGGACTGTTG	57°C	1012	27	This study

Table 2. Characteristics of CL patients included in the study

No.	Initials	Code	Sex	Age	Clinical Form	Lesion(S) Location	Species	ST
1	FAKE	24	F	59	Atypical	Face	<i>L.m</i>	1
2	AHKA	25	M	20	Atypical	Hand	<i>L.m</i>	1
3	IRHA	26	F	53	Atypical	Hand	<i>L.m</i>	7
4	SAHO	08	M	30	Atypical	Hand	<i>L.t</i>	15
5	MEKH	21	F	61	Atypical	Foot	<i>L.t</i>	12
6	FAKA	27	F	42	Atypical	Hand	<i>L.m</i>	3
7	MAHO	22	F	32	Atypical	Foot	<i>L.t</i>	10
8	SHSA	28	F	16	Atypical	Face	<i>L.m</i>	13
9	FAGH	30	F	61	Atypical	Hand	<i>L.m</i>	1
10	MAAK	32	F	38	Atypical	Hand	<i>L.m</i>	2
11	HEAB	33	M	61	Atypical	Hand-Foot	<i>L.m</i>	3
12	HESA	34	M	70	Atypical	Hand	<i>L.m</i>	3
13	RAFA	36	M	61	Atypical	Foot	<i>L.m</i>	1
14	FESO	37	F	47	Atypical	Hand	<i>L.m</i>	3
15	GHRE	23	M	75	Atypical	Hand	<i>L.t</i>	9
16	ALHA	39	M	54	Atypical	Foot	<i>L.m</i>	2
17	AHFA	31	M	32	Atypical	Hand	<i>L.t</i>	8
18	AGGH	41	F	62	Atypical	Hand-Foot	<i>L.m</i>	1
19	SAMO	42	F	48	Atypical	Hand	<i>L.m</i>	12
20	FAAR	50	M	24	Atypical	Hand-Foot	<i>L.m</i>	1
21	SAHO	35	M	58	Atypical	Hand	<i>L.t</i>	11
22	FAAL	53	M	1	Typical local lesion	Foot	<i>L.m</i>	3
23	ZIMA	43	F	44	Typical local lesion	Hand	<i>L.t</i>	6
24	MERE	54	M	29	Typical local lesion	Hand-Foot	<i>L.m</i>	1
25	AMAS	55	F	63	Typical local lesion	Hand-Foot	<i>L.m</i>	1
26	NAAR	56	F	3	Typical local lesion	Face	<i>L.m</i>	1
27	ZAGH	46	F	23	Typical local ulcer	Hand	<i>L.t</i>	16
28	ALVA	57	M	58	Typical local lesion	Hand	<i>L.m</i>	1
29	NEAH	47	F	62	Typical local lesion	Hand	<i>L.t</i>	17
30	REHE	58	M	49	Typical local lesion	Hand	<i>L.m</i>	1
31	NITA	59	F	6	Typical local lesion	Face-Hand-Trunk	<i>L.m</i>	4
32	HANU	60	F	1	Typical local lesion	Face	<i>L.m</i>	1
33	MOTA	51	F	55	Typical local lesion	Face	<i>L.t</i>	1
34	ERNA	62	M	17	Typical local lesion	Trunk	<i>L.m</i>	5
35	NAJA	63	M	66	Typical local lesion	Hand	<i>L.t</i>	1
36	FAFA	64	M	43	Typical local lesion	Hand	<i>L.m</i>	1
37	AZLA	65	F	31	Typical local lesion	Hand-Trunk	<i>L.m</i>	1
38	MEAL	67	F	38	Typical local lesion	Face	<i>L.t</i>	1
39	ADAR	68	M	33	Typical local lesion	Foot	<i>L.m</i>	1
40	SOSA	69	F	30	Typical local lesion	Hand	<i>L.t</i>	1
41	ZAAR	70	F	46	Typical local lesion	Hand	<i>L.m</i>	1

Table 3. Heterozygous nucleotide sites of seven enzyme genes in *L. major* and *L. tropica* strains

Target	<i>L. major</i>			<i>L. tropica</i>		
	Code	Heterozygous site	Total sites per gene	Code	Heterozygous site	Total sites per gene
<i>6pgd</i>	26	234 A=G	7	21, 22, 23, 31, 35	762 C=T; 775 A=C; 818 C=A; 952 C=T; 994 T=C; 1194 A=C	6
	28, 42	98 C=T; 112 C=A; 154 A=C; 288 G=T; 303 A=G; 528 A=G				
<i>asat</i>	28	231 T=C; 303 G=A; 376 G=T; 520 G=T; 576 G=T; 648 G=A; 618 G=C	7	35	361 A=G; 362 A=G; 384 G=A	5
				47	438 C=T; 439 T=A	
<i>fh</i>	42	38 C=T; 53 G=C 90 C=T; 105 G=C; 488 G=A; 540 A=G	6	21, 35	88 C=T; 103 C=G; 538 A=G	3
<i>g6pd</i>	28	124 G=A; 144 A=G; 240 T=C; 409 A=G; 615 T=C	5	21, 22, 23, 35	178 G=A; 198 G=A; 294 T=C; 463 A=G; 669 C=T	6
				31	178 A=G; 198 G=A; 294 T=C; 457 G=C;	
<i>icd</i>	42	156 T=C; 186 T=C; 376 T=C; 614 T=C	4	21, 22, 23, 31, 35, 46	453 T=C; 483 T=C; 673 T=C; 911 T=C	4
<i>mpi</i>	36	147 C=G 95 G=C	2			
<i>trys</i>	28	529 A=G; 539 G=A; 548 A=G	3	21, 22, 31, 35, 46	442 G=A; 451 A=G	2

	Marker						ST	Frequency (%)
	<i>6pgd</i>	<i>asat</i>	<i>fh</i>	<i>g6pd</i>	<i>icd</i>	<i>mpi</i>		
1	1	1	1	1	1	1	1	24.39
2	1	1	1	1	1	1	2	2.44
3	1	1	1	1	1	1	3	6.10
3	2	1	2	1	1	2	4	1.22
3	3	1	2	1	1	2	5	1.22
3	3	1	2	1	1	3	6	1.22
4	3	1	2	1	1	4	7	1.22
5	3	2	3	1	1	4	8	1.22
5	4	3	4	1	1	5	9	1.22
5	5	3	4	2	2	6	10	1.22
5	6	4	4	2	2	7	11	1.22
5	3	4	4	2	2	7	12	2.44
5	3	4	5	2	2	7	13	1.22
6	3	4	6	3	3	7	14	1.22
6	1	4	6	3	3	5	15	1.22
6	1	4	6	4	3	5	16	1.22
6	1	4	6	5	3	5	17	1.22
6	7	5	7	1	1	1	18	1.22
7	8	6	8	1	4	2	19	1.22
7	9	7	9	4	5	8	20	1.22
7	10	7	10	5	4	9	21	1.22
8	11	8	11	6	6	8	22	1.22
7	11	9	12	7	7	8	23	1.22
9	10	10	11	8	8	8	24	1.22
3	12	11	13	9	9	10	25	1.22
10	12	12	13	10	10	11	26	1.22
11	12	1	13	10	1	12	27	1.22
12	12	1	13	10	11	5	28	1.22
12	12	1	13	10	11	13	29	1.22
13	12	5	14	11	12	14	30	1.22
14	12	13	15	11	11	14	31	1.22
15	13	14	16	12	11	14	32	1.22
16	14	15	17	11	11	15	33	1.22
17	14	14	17	11	12	15	34	1.22
18	14	14	17	13	13	16	35	1.22
19	14	16	17	11	14	17	36	1.22
20	1	17	18	11	14	17	37	1.22
21	1	18	1	14	15	18	38	1.22
22	1	19	2	15	12	16	39	1.22
23	15	19	19	10	12	19	40	1.22
23	15	14	20	16	16	20	41	1.22
24	16	11	11	16	16	21	42	1.22
25	17	11	21	17	16	22	43	1.22
23	5	11	6	18	17	23	44	1.22
26	18	20	6	2	17	1	45	1.22
27	19	20	22	5	18	16	46	1.22
8	7	20	23	11	16	18	47	1.22
28	8	21	9	19	19	14	48	1.22
29	8	20	24	11	20	24	49	1.22
30	20	22	14	20	21	25	50	1.22
31	21	20	25	8	1	26	51	1.22
32	22	20	2	11	22	27	52	1.22
33	16	22	19	21	23	11	53	1.22
34	5	20	20	17	24	12	54	1.22

35	3	20	26	22	25	12	55	1.22
36	3	23	27	23	26	28	56	1.22
37	23	24	28	24	27	8	57	1.22
1	23	25	28	25	26	8	58	1.22
169	321	260	358	299	346	228	Total no. of polymorphism	

Table 4. Allelic profiles and sequence types (STs) of all strains of *Leishmania* included in the study

Table 5. Genetic diversity indices of *L. major* and *L. tropica* clinical isolates

Marker	S and Eta	H	Hd	±SD	Pi (π)	θ	dN/dS	k	Selected region
<i>6pgd</i>	19	8	0.743	± 0.082	0.01755	0.01249	191.67/72.33	7.929	1-1440
<i>asat</i>	15	7	0.45	± 0.151	0.00835	0.00948	351.17/122.83	3.983	1-1298
<i>fh</i>	14	5	0.714	± 0.127	0.01133	0.00809	494.17/ 207.83	8	1-1658
<i>g6pd</i>	16	6	0.745	± 0.079	0.01653	0.01146	296.17/105.83	6.712	1-1691
<i>icd</i>	19	5	0.58	± 0.099	0.02206	0.01305	285.17/101.83	8.605	1-1280
<i>mpi</i>	9	4	0.575	± 0.115	0.01497	0.01005	199.50/70.50	4.042	1-1267
<i>trys</i>	14	4	0.658	± 0.065	0.01255	0.00822	349.00/128.00	6.026	1-1003

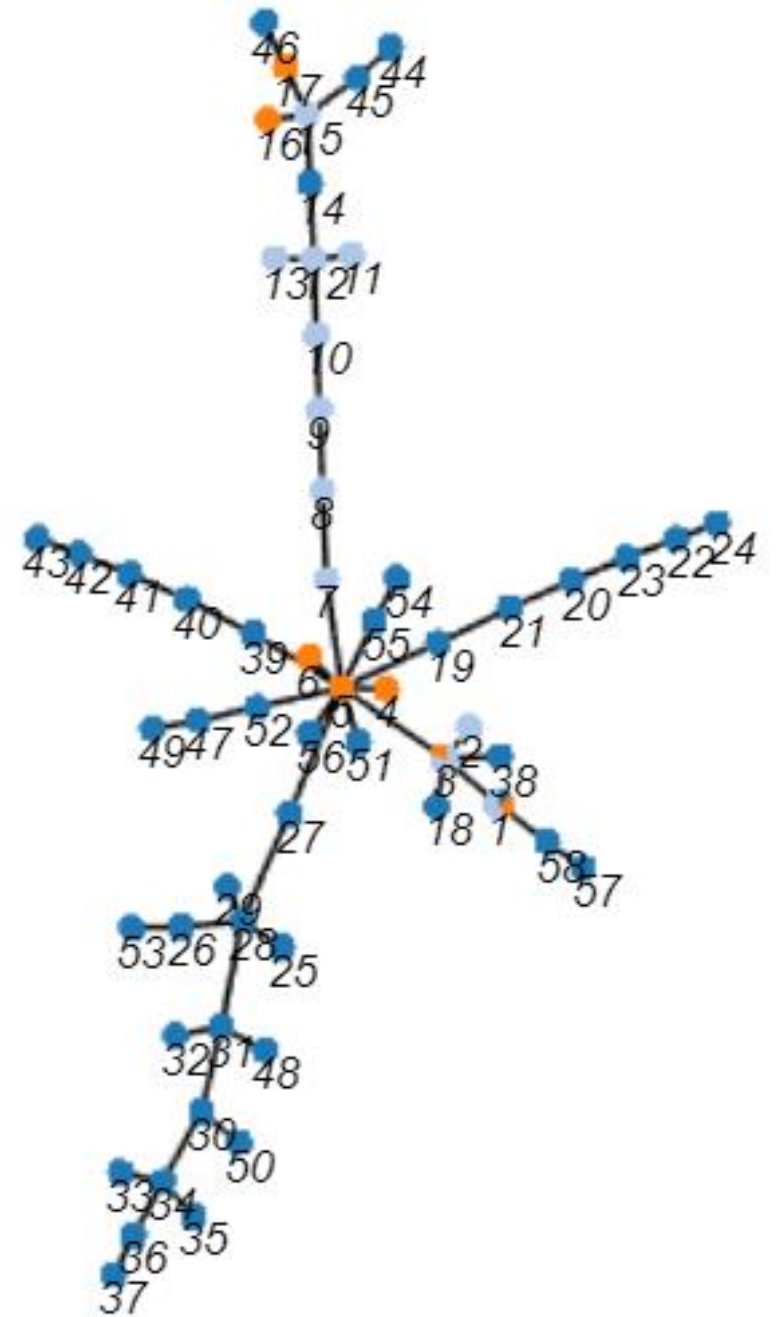
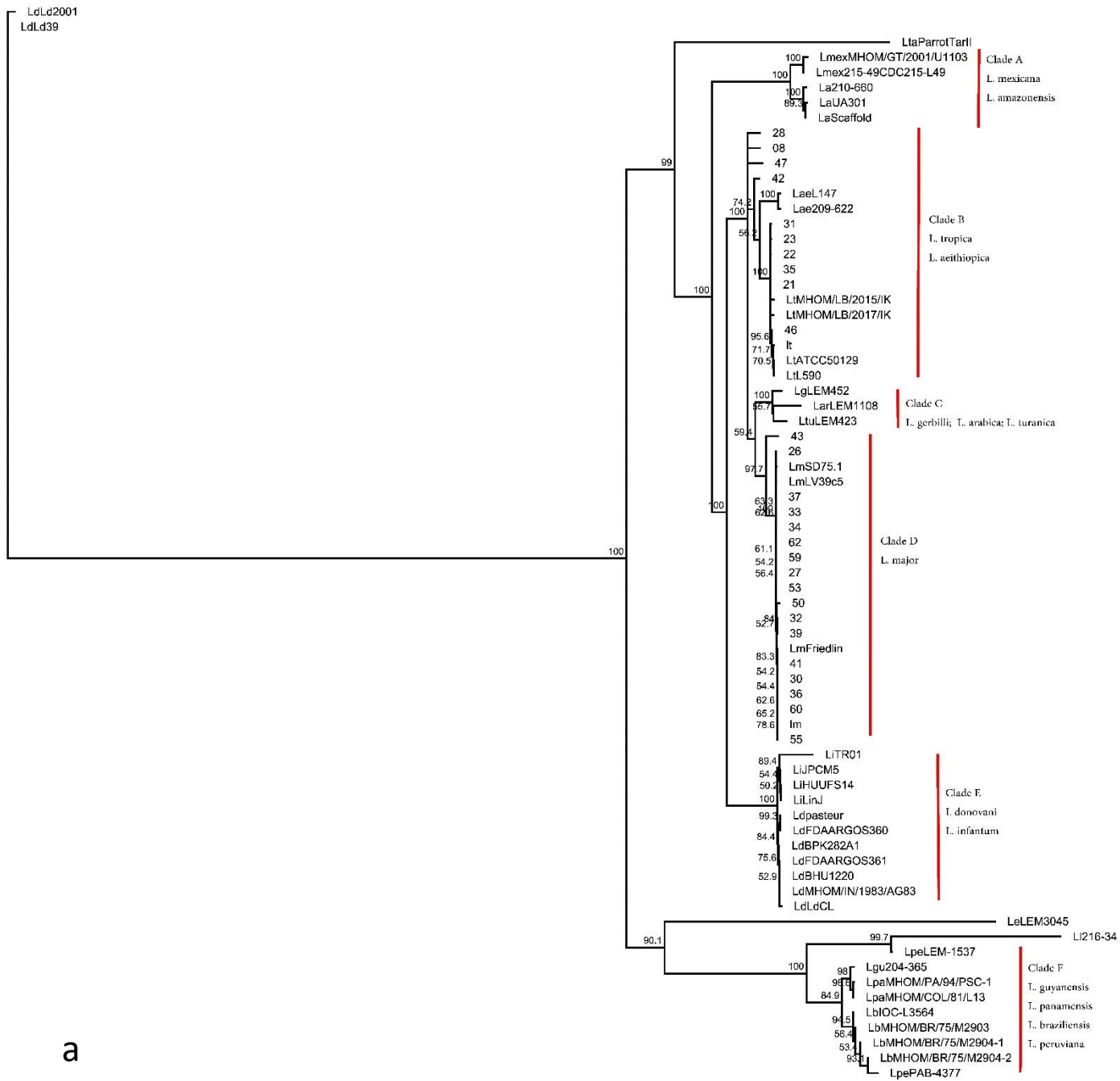
Abbreviations: S number of polymorphic sites, Eta total number of mutations, Hd haplotype diversity, π nucleotide diversity. The mutation parameter (θ) is defined as $4Nm$ for autosomal loci of diploid organisms, where N is the effective population size (diploid individuals) and m is the neutral mutation rate (per gene or per base pair) per generation. K average number of nucleotide differences.

Table 6. Genetic diversity indices of *Leishmania* strains isolated from typical CL vs. atypical CL patients

Marker	Typical CL		Atypical CL	
	Haplotype Diversity (Hd)±SD	Nucleotide diversity (π)	Haplotype diversity (Hd) ±SD	Nucleotide diversity (π)
<i>6pgd</i>	0.661±0.215	0.00922	0.788±0.050	0.01369
<i>asat</i>	0.667± 0.314	0.00170	0.641±0.150	0.01230
<i>trys</i>	0.867±0.129	0.01149	0.850±0.048	0.01140
<i>g6pd</i>	0.833±0.222	0.01455	0.819±0.050	0.01374
<i>mpi</i>	0.500±0.265	0.01662	0.530±0.076	0.01967
<i>icd</i>	0.600±0.215	0.01910	0.714±0.048	0.01881
<i>fh</i>	0.667± 0.314	0.01139	0.905±0.103	0.00618
Average	0.685±0.239	0.01201	0.749±0.750	0.013684

Figure legend

Figure 1. Phylogenetic reconstruction based on the seven gene markers of the MLST scheme. **a)** Neighbor-Joining phylogenetic tree cladogram of concatenated sequences of *Leishmania*. List of strains retrieved from databases is described in supporting file 2. Numbers show the strains codes isolated from CL patients in this study as described in Table 2. Im and It are reference strains. Numbers above branches represent bootstrap support. **b)** The eBURST algorithm was employed to evaluate the relationships between 58 STs, corresponding to all *Leishmania* strains included in analysis using the seven gene markers of the MLST scheme. Clonal complex showing the relationship between STs further categorized based on the clinical type of CL as shown by blue nodes (strains from atypical CL) and orange nodes (strains from typical CL).



a

b